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13. SUPPLEMENTARY NOTES

14. ABSTRACT
KAI1 is a tumor metastases suppressor gene which is capable of blocking the metastatic process without affecting the primary tumorigenesis. In this project we tested the hypothesize that the KAI1 protein on tumor cells interacts with gp-Fy (DARC) on the endothelial cells, which activates a signal pathway of the KAI1 molecule, and that this activation eventually leads to cell growth arrest of tumor cells. We originally planned to examined whether the interaction of KAI1 and DARC leads to suppression of tumor metastasis in vivo (Task 1), and identify specific peptide sequences that activate KAI1 and to assess the efficacy of the peptides on tumor growth in an animal model (Task 2). We have successfully completed Task 1 and published the results in Nature Medicine. Task 2 has been partly accomplished but there are some tasks remained. However, we consider that our project has been overall quite successful and our original idea has been fully developed. The results of this project have led to successful R01 funding, and we will continue to develop and expand our research.

15. SUBJECT TERMS
` Tumor metastases suppressor, prostate cancer, tumorigenesis`

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INTRODUCTION

The KAI1 gene was originally isolated as a prostate-specific tumor metastasis suppressor gene, using the microcell-mediated chromosome transfer method (1). Ample evidence from both clinical data and the results of in vitro as well as animal experiments overwhelmingly support the notion that KAI1 is a metastasis suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis (2). Based on our preliminary data, we hypothesize that the KAI1 protein on tumor cells interacts with DARC (gp-FY) on the endothelial cells, which activates a signal pathway of the KAI1 molecule, and that this activation eventually leads to cell growth arrest of tumor cells. To test our hypothesis, we proposed to examine whether the interaction of KAI1 and DARC leads to suppression of tumor metastasis in vivo (**Task 1**), and identify specific peptide sequences that activate KAI1 and assess the efficacy of the peptides on tumor growth in an animal model (**Task 2**). Our long-term goal is to elucidate the molecular mechanism of tumor suppression by the KAI1 gene and to develop an effective therapeutic method which restores the function of the KAI1 gene in the metastatic tumor cells.

BODY

Task 1-a. (completed)
Prepare endothelial cells from a DARC knockout and wild type mouse and test their ability to bind and kill tumor cells that express KAI1

As reported last year, we have completed this task and published the results in Nature Medicine (Nature Medicine, 12, 933). As shown in Fig. 2 in this article, we have extensively examined the ability of cancer cell to bind to DARC on the endothelial cells. The interaction between Kai1 and DARC blocked tumor cell growth and led them to senescence. We also found that the senescence is induced through activation of TBX2 and p21. To further extend our observation, we are currently using a phospho-specific signal array as well as a gene microarray to identify a specific target which ultimately blocks the metastasis process in response to a signal activated by the interaction of DARC and Kai1.

Task1-b. (completed)
Examine the effects of siRNA against the DARC gene on the binding and killing ability of KAI1 to endothelial cells

We have tested synthetic siRNA to block the expression of DARC in endothelial cells. However, the transfection efficiency for primary culture of endothelial cells had been unexpectedly poor even though we have used several different commercial transfection kits. We then switch to an shRNA expression system using adenovirus vector. However, we also encountered technical difficulties in constructing an effective vector, and the efficiency of shRNA expression was very poor. Accordingly, we abandoned this approach and shifted our effort to in vivo experiments using DARC knockout mice. Our results clearly indicate that the knock-out of DARC nullified the anti-metastatic effect of Kai1 in these mice, suggesting that the DARC-Kai1 interaction indeed essential for tumor growth arrest.

Task 1-c. (completed)
Examine the metastatic ability of a spontaneously developing tumor in DARC knockout mice by constructing a hybrid animal between a transgenic prostate tumor mouse, TRAMP, and the DARC knockout mouse

Because the doing experiments with a spontaneous mouse model takes a time, we switched to a xenograft model using DARC knockout mouse. We examined the degree of tumor metastases of transplanted tumor cells with or without expression of Kai1 in the DARC knockout mice. As our hypothesis predicted, the degree of metastases dramatically reduced when we transplanted tumor cells with Kai1 in the DARC knockout mouse. These results were published during this reporting cycle (Nature Medicine, 12, 933).

Therefore, we consider that Task 1 has been successfully accomplished.

Task 2-a. (completed)
Construct a series of deletions of DARC and test their binding ability to KAI1.

We have completed this task. We tested several individual domains as well as serial deletions from the N-terminus of the KAI1 gene against full length DARC target and vice versa. Our results indicate that the first 32 amino acids from the N-terminus of KAI1, spanning the first intracellular and transmembrane domains, are dispensable for the interaction, but the conformation of the protein as a whole may be important as none of the other fragments yielded a positive interaction. On the other hand, deletion of the first extracellular domain of DARC at N-terminus completely abrogated the interaction, suggesting that the N terminus of DARC is essential for binding to KAI1.

Task 2-b.
Screening a phage display library followed by sequencing the interacting clone

This task is incomplete. We have obtained a phage-display library (Biolab) and have screened the library by first adsorbing the phages to Kai-1 negative cell line followed by recovering the phages that did not attach to these cells. The recovered phages were again adsorbed to Kai+ cells, and phages adsorbed to these cells were recovered. By panning these phages, we isolated 24 different phage clones. We did performed several experiments to confirm our results in vitro. However, the results were not conclusive. We then switched our phage-display library to another source but again the results were not conclusive. We are currently exploring a possibility that the interaction of KAI1 with DARC requires a co-receptor.

Task 2-c.
Synthesize small peptides corresponding to the DARC binding domain and test for their tumor suppressive activity

Based on the results of Task 2a, we synthesized two different peptides that cover the essential 32 amino acids region for the DARC-Kai1 interaction. We first tested in vitro to see if these

peptides interfere with the interaction of DARC and Kai1. However, we found that these peptides did not show any effect on tumor cells. Because another group found that an 18-amino acid peptide corresponding to the N-terminal has biological activity, we also tested this peptide. However, again it did not show any growth inhibitory effect on Kai1+ tumor cells. Although we can synthesize various peptides in this region, it is not a cost effective approach. Therefore, we decided to focus our effort on Task 2-b, so that the results of the experiments will provide us with more options for approach.

Task 2-d.
Test the efficacy of the specific peptides in SCID mice model of prostate cancer

We have not pursued this task because we need to wait for the results of Task 2-c.

KEY RESEARCH ACCOMPLISHMENTS

1. We provided convincing evidence to show that Kai1 interacts with DARC on the endothelial cells both in vitro and in vivo. We also found that this interaction induce cell senescence by activation of TBX and the p21 genes.
2. We found that the interaction of Kai1 and DARC is essential to block metastasis in vivo.
3. We narrowed down the region of interaction between DARC and KAI1 in vitro.
4. We were able to publish the results of Task 1 in the high-impact journal, Nature Medicine.

REPORTABLE OUTCOMES

Peer reviewed publications

1. Bandyopadhyay, S., Zhan, R., Chaudhuri, M., Watabe, M., Pai, S.K., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Takano, Y., Saito, K, Pauza, M., Hayashi, S., Wang, Y., Mohinta, S., Mashimo, T., M. Iiizumi, Furuta, E. and Watabe, K. (2006) Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. Nature Medicine. 12, 933-938.
2. Bandyopadhyay, S and Watabe, K. (2007) The Tumor Metastasis Suppressor Gene Drg-1 in Cancer Progression and metastasis in "Developments in Metastasis Suppressors" Nova Publishers.
3. Iiizumi, M., Bandyopadhyay, S. and Watabe, K. (2007) Interaction of DARC and KAI1: a critical step in metastasis suppression. Cancer Research. 15, 1411-14
4. Megumi Iiizumi, Sonia Mohinta, Sucharita Bandyopadhyay and Kounosuke Watabe. (2007) Tumor - endothelial cell interactions: Therapeutic potential. Microvascular Research. 74, 114-120

Grant funding

Based on the data we have obtained from this project, we applied for R01 to NIH. We received an excellent review and the proposal was funded without any revision.

1R01CA129000 Watabe (PI)   NIH 9/1/07-8/31/12
 "Functional role of tumor metastasis suppressor gene, KAI1, in tumor progression"

Abstract/presentation

1. Watabe, K. (2006) The role of KAI1 and Drg1 in metastases suppression. BenMay
   symposium. Chicago
2. Watabe, K. (2006) Metastasis suppressor genes, Kai11 and Drg1. Tokyo University, Japan.
   Invited lecture
3. Watabe, K. (2006) The role of metastases suppressor genes in prostate cancer. Kyoto
   University, Japan. Invited lecture
4. Watabe K (2007) Functions of metastasis suppressor genes, NDRG1 and KAI1. University of
   Chicago, Medical School Chicago IL.
5. Watabe, K (2007) Interaction of KAI1 On Tumor Cells with DARC On Vascular
   Endothelium Leads to Metastasis Suppression. IMPCT meeting, Atlanta, Ga

Employment

1. Dr. Megumi Iiizumi (Postdoc) has been supported by the current grant.
2. Sonia Mohinta (currently in Ph.D. program) has been partly supported by the current grant.

CONCLUSIONS

We have successfully completed all experiment proposed in Task 1, and the results were
published in Nature Medicine. The paper was cited in two review articles in Nature Medicine
and in Cancer Cell. We believe that this conceptual breakthrough about the mechanism of
metastases suppression opened a new avenue for the tumor metastases research and also
provided with a novel approach for the treatment of prostate cancer. As an extension of this
project, we have applied for R01 to NIH based on the data obtained from this project. The
application was well received by the review panel and the proposal was funded at the first round
without revision. Although some tasks have not been completed, we consider the project of this
idea development award has been successfully completed.

So what?
We believe that the progress we have made so far presents a breakthrough concept in the field of
tumor metastases. The fact that the interaction between Kai1 and DARC results in cell
senescence through the activation of p21 and TBX provides a bases of new target-specific
approach to treat prostate cancer.   This project will be carried over to the next R01 project in
which we will further investigate the signal mechanism of KAI/DARC interaction in depth.

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   chromosome 11p11.2. *Science* 268, 884-886 (1995).

2. Yoshida, B.A., Sokoloff, M.M., Welch, D.R. & Rinker-Schaeffer, C.W. Metastasis-suppressor genes: a review and perspective on an emerging field. *J Natl Cancer Inst.* 92,1717-1730 (2000).

Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression

Sucharita Bandyopadhyay[1], Rui Zhan[1], Asok Chaudhuri[2], Misako Watabe[1], Sudha K Pai[1], Shigeru Hirota[3], Sadahiro Hosobe[3], Taisei Tsukada[3], Kunio Miura[3], Yukio Takano[3], Ken Saito[3], Mary E Pauza[1], Sunao Hayashi[1], Ying Wang[1], Sonia Mohinta[1], Tomoyuki Mashimo[1], Megumi Iiizumi[1], Eiji Furuta[1] & Kounosuke Watabe[1]

CD82, also known as KAI1, was recently identified as a prostate cancer metastasis suppressor gene on human chromosome 11p1.2 (ref. 1). The product of CD82 is KAI1, a 40- to 75-kDa tetraspanin cell-surface protein also known as the leukocyte cell-surface marker CD82 (refs. 1,2). Downregulation of KAI1 has been found to be clinically associated with metastatic progression in a variety of cancers, whereas overexpression of CD82 specifically suppresses tumor metastasis in various animal models[3]. To define the mechanism of action of KAI1, we used a yeast two-hybrid screen and identified an endothelial cell-surface protein, DARC (also known as gp-Fy), as an interacting partner of KAI1. Our results indicate that the cancer cells expressing KAI1 attach to vascular endothelial cells through direct interaction between KAI1 and DARC, and that this interaction leads to inhibition of tumor cell proliferation and induction of senescence by modulating the expression of TBX2 and p21. Furthermore, the metastasis-suppression activity of KAI1 was significantly compromised in DARC knockout mice, whereas KAI1 completely abrogated pulmonary metastasis in wild-type and heterozygous littermates. These results provide direct evidence that DARC is essential for the function of CD82 as a suppressor of metastasis.

We screened the human normal prostate cDNA library using the full-length *CD82* cDNA as bait in a yeast two-hybrid interaction trap[4] and identified Duffy antigen receptor for chemokines (DARC, also known as gp-Fy and encoded by *DARC*) as a potential interactor for KAI1. A liquid β-galactosidase assay quantitatively showed the strength and specificity of the interaction between KAI1 and DARC (**Fig. 1a**). DARC is an approximately 45-kDa, seven-transmembrane protein expressed on vascular endothelium of various organs, as well as on red blood cells and certain epithelial cells[5,6]. It binds chemokines of both C-C and C-X-C families, although ligand binding by DARC does not induce G-protein-coupled signal transduction or Ca^{2+} flux[7,8]. The *DARC* gene has two alleles, Fya and Fyb, which differ only at amino acid residue 44 (ref. 9). Sequence analysis showed that the cloned

DNA identified by our screening represents the spliced isoform of the Fyb allele of *DARC* (**Fig. 1b**). To examine the interaction of KAI1 and DARC in mammalian cells, we carried out a coimmunoprecipitation experiment using a highly metastatic prostate carcinoma cell line, AT6.1, which was stably transfected with a Flag epitope–tagged *DARC* gene. The cells were then transiently transfected with a hemagglutinin (HA)-tagged KAI1 plasmid, and the cell lysate was incubated with antibody to Flag. We found that KAI1 coprecipitated with Flag-tagged DARC, suggesting that KAI1 can interact with DARC in mammalian cells (**Fig. 1c**). To localize the regions of KAI1 and DARC that are essential for this interaction, we tested individual domains as well as serial deletions from the amino terminus of KAI1 against full-length DARC target and vice versa in yeast mating assay. Our results indicate that the first intracellular and transmembrane domains of KAI1 are dispensable for this interaction (**Fig. 1d**). On the other hand, deletion of the first extracellular domain of DARC at the amino terminus completely abrogated the interaction, suggesting that the amino terminus of DARC is essential for binding to KAI1 (data not shown).

To assess the relevance of the interaction between KAI1 and DARC, we next examined the localization of DARC in prostate cancer tissue by immunohistochemistry. We found that DARC is highly expressed in the prostate endothelium, particularly in the small veins and venules, as well as in lymphatic vessels, whereas it was undetectable in the epithelial cells and stroma (**Fig. 1e**). The expression of DARC in endothelium was found to be essentially the same in normal, hyperplastic glands and high-grade carcinomas. We observed a similar pattern of expression of DARC in breast and lung cancer samples (data not shown). On the other hand, KAI1 is highly expressed in the normal epithelial cells in these organs, and its expression is substantially reduced in carcinoma, as reported previously[3]. Because expression of *DARC* in these organs is restricted to the vasculature, it is unlikely that KAI1 on epithelial cells interacts with DARC protein in the same cell. Instead, it suggests that such an interaction takes place when cancer cells expressing KAI1 intravasate and encounter the endothelial lining of small blood vessels. Consistent with this hypothesis, a previous study using epifluorescence microscopy detected

[1]Southern Illinois University School of Medicine, Department of Medical Microbiology, Immunology and Cell Biology, 801 N. Rutledge Street, PO Box 19626, Springfield, Illinois 62794-9626, USA. [2]Lindsley F. Kimball Research Institute of the New York Blood Center, 310 E. 67th St., New York, New York 10021, USA. [3]Akita Red Cross Hospital, 222-1 Saruta, Kitakamide, Akita City, Japan 010-1495. Correspondence should be addressed to K.W. (kwatabe@siumed.edu).

Figure 1 KAI1 interacts with DARC *in vitro*. (**a**) Quantification of interaction between KAI1 and DARC. Yeast cells transformed with an appropriate combination of expression plasmids were grown in minimal medium in the presence of glucose (white bar) or galactose (black bar) as indicated. The β-galactosidase activity is expressed in Miller units (U). pBait and pTarget are a pair of positive control interactors provided by the manufacturer. (**b**) Alleles and splice variants of *DARC*. The junctions of two exons in the biexonic isoforms (#2, #4) are indicated by arrows. (**c**) Coimmunoprecipitation of DARC and KAI1 in mammalian cells. AT6.1/Flag-DARC permanent clone or the parental cell line was tested for DARC expression by immuno-precipitation with monoclonal antibody to Flag covalently crosslinked to agarose beads followed by western blot with monoclonal antibody to Flag (lanes 1, 2). For coimmunoprecipitation, AT6.1/Flag-DARC cells were transiently transfected with HA-tagged KAI1 expression plasmid, proteins were pulled down by Flag-specific agarose beads and KAI1 was detected by western blot with antibody to hemagglutinin (lane 3). To confirm the HA-KAI1 position, the AT6.1/Flag-DARC cells

were transfected with HA-KAI1 as above and immunoprecipitation and western bolt were performed with monoclonal antibody to hemagglutinin and protein G agarose followed by western blot with the same monoclonal antibody (lane 5). AT6.1/Flag-DARC cells without KAI1 transfection or parental AT6.1 cells served as negative controls (lanes 4, 6). IgH appeared in lanes 5 and 6, as antibody to hemagglutinin was not crosslinked to the agarose beads during immunoprecipitation. (**d**) Analysis of interactions of various domains of KAI1 with DARC. Regions of KAI1, as indicated by the amino acid sequence numbers, were tested: '+' indicates positive interaction and '–' indicates lack of interaction. (**e**) DARC is expressed only in the vascular endothelium of prostate tissue. Immunohistochemistry was performed on clinical samples using the polyclonal antibody to DARC. Representative fields of normal prostate gland and various grades of prostate carcinoma are shown in the upper panel. DARC is detectable only in the vascular endothelium and red blood cells (RBC). The lower panel represents a magnified view of a blood vessel from a high-grade cancer section.
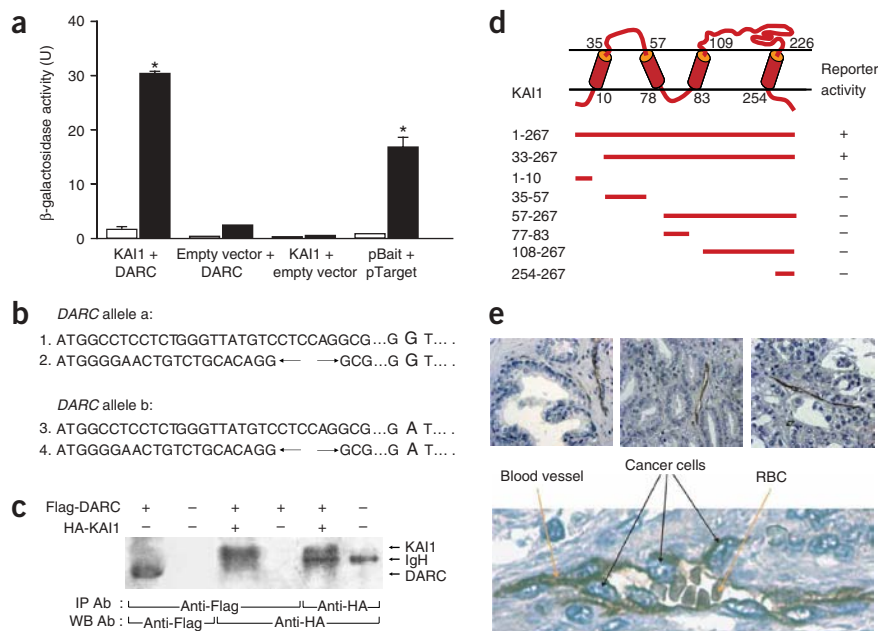
metastatic tumor cells attached to the endothelium of precapillary arterioles and capillaries in intact mouse lungs[10]. In agreement with this observation, in our archive of specimens, examination of small blood vessels in a high-grade cancer area indicated that cancer cells are often attached to endothelium of blood vessels (**Fig. 1e**).

We next tested the possibility that KAI1 on tumor cells interacts with DARC on endothelial cells by performing a cell-to-cell binding assay *in vitro* in which green fluorescent protein (GFP)-tagged AT6.1 (KAI1−) or AT6.1/Flag-KAI1 (KAI1+) cells were overlaid on DARC+ endothelial cells, human bone marrow endothelial cells (HBMEs) and human umbilical vein endothelial cells (HUVECs). We observed a significantly higher percentage of attachment of KAI1+ cells compared with KAI1− cells to both types of endothelial cells in a time-dependent manner. Moreover, antibody to KAI1 abrogated this binding, indicating the direct involvement of KAI1 in the process (**Fig. 2a**). We next carried out the same binding assay by overlaying the tumor cells on AT6.1 cells with or without expression of DARC. KAI1+ tumor cells exhibited a binding affinity specifically to the DARC+ AT6.1/Flag-Fy cells (**Fig. 2a**), confirming that the binding of KAI1+ cells to these endothelial cells is indeed due to the expression of DARC. To show a direct interaction between these two membrane proteins in a cell-to-cell manner, we mixed the KAI1+ tumor cells HT-38 and DARC+ HUVECs in the presence of the membrane-impermeable cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP), lysed the cells and performed a coimmunoprecipitation experiment. KAI1 coprecipitated with DARC (**Fig. 2b**), whereas another tetraspanin (CD81) did not, indicating a specific interaction between KAI1 and DARC. These results indicate that KAI1-expressing tumor cells can bind to endothelial cells via the interaction between KAI1 and DARC, and suggest the possibility that the metastasis suppressor function of KAI1

is partly due to the trapping of the tumor cells on the endothelial linings of vessels.

It was previously reported that treatment of Jurkat cells with a monoclonal antibody to KAI1 inhibited proliferation of the cells *in vitro*[11]. Therefore, we sought to determine whether this antibody would elicit a similar response in tumor cells expressing KAI1. We found that this antibody significantly inhibited DNA synthesis in KAI1+ prostate tumor cells (**Fig. 2c**). We also obtained similar results for the breast and lung carcinoma cell lines MDA-MB-435 and A549, respectively (data not shown). These results suggest that the growth of KAI1-expressing tumor cells is suppressed when KAI1 on the tumor cell surface is engaged by an appropriate ligand. Consistent with this idea, it was previously reported that exposure of prostate tumor cells to nerve growth factor led to upregulation of KAI1, which was also associated with downregulation of cell proliferation *in vitro*[12]. To examine whether the signaling pathway leading to growth arrest of tumor cells is also activated when KAI1 binds to DARC, we measured the rate of DNA synthesis in tumor cells when they were allowed to contact cells that either did or did not express DARC. The rate of DNA synthesis was significantly reduced only when the cells expressing KAI1 (AT6.1/Flag-KAI1) contacted the DARC+ endothelial cells (HBMEs or HUVECs) or the prostate carcinoma cell line (AT6.1/Flag-DARC; **Fig. 2d**). We obtained similar results for the breast and lung carcinoma cell lines MDA-MB-435 and A549, respectively (data not shown).

To further corroborate the notion of growth arrest of tumor cells upon interaction with DARC on the endothelial cell surface, we mixed GFP-tagged AT6.1 and AT6.1/Flag-KAI1 cells with HBMEs or HUVECs and then selected for GFP+ tumor cells. We found that the ability of tumor cells to form colonies significantly decreased when

AT6.1/Flag-KAI1 cells (KAI1+), compared with AT6.1 cells (KAI1−), interacted with HBMEs or HUVECs (**Fig. 2e**). We confirmed that this effect is mediated by DARC in the endothelial cells by performing similar experiments in which AT6.1/Flag-KAI1 or AT6.1 cells were mixed with cells with or without DARC expression (AT6.1/Flag-DARC or AT6.1; **Fig. 2e**). Therefore, our data suggest that the interaction between KAI1 and DARC leads to a growth-suppressive effect on the KAI1-bearing cell; thus, the status of KAI1 expression on tumor cells has a key role in determining their fate once they intravasate into the blood vessels.

To examine whether the interaction between KAI1 and DARC is essential for the metastasis suppressor function of KAI1 *in vivo*, we used *Darc*−/− mice[13]. We chose the syngenic metastatic tumor cell lines B16BL6 and B16F10 to establish tumors in these mice and generated several KAI1+ clones or empty-vector transfectants in these cells (**Fig. 3a**). We then injected the B16BL6 derivatives subcutaneously into *Darc*−/− mice and heterozygous and wild-type littermates. We found that primary tumors developed in all mice. The growth rate and final volume of tumors did not significantly vary with the KAI1 level in the tumor cells or with DARC status of the mice (**Table 1**). The

KAI1+ clones, however, developed significant numbers of pulmonary metastases in *Darc*−/− mice, whereas metastasis was almost completely abrogated when the same clones were injected in the heterozygous and wild-type littermates (**Fig. 3b** and **Table 1**). The tumor cells lacking KAI1 (B16BL6/vector), however, metastasized equally in all three groups of mice. Thus, in the absence of DARC, even the tumor cells expressing large amounts of KAI1 recapitulated the metastatic phenotype of downregulation of *CD82*. To further corroborate the effect of DARC on the metastatic ability of KAI1-bearing cells, we used an experimental metastasis model in which the metastatic cell line B16F10 stably transfected with KAI1 expression plasmid or an empty vector was injected intravenously into *Darc*−/− mice and their control littermates. The KAI1+ clones resulted in a significantly higher number of pulmonary metastases in the DARC knockout mice, whereas the empty vector transfectant metastasized regardless of the DARC status of the host (**Table 1**). These results support our hypothesis that DARC has a crucial role in the metastasis suppressor function of KAI1 *in vivo*.

DARC is known to be a promiscuous chemokine receptor; however, our *in vitro* data indicate that this function of DARC is not likely to
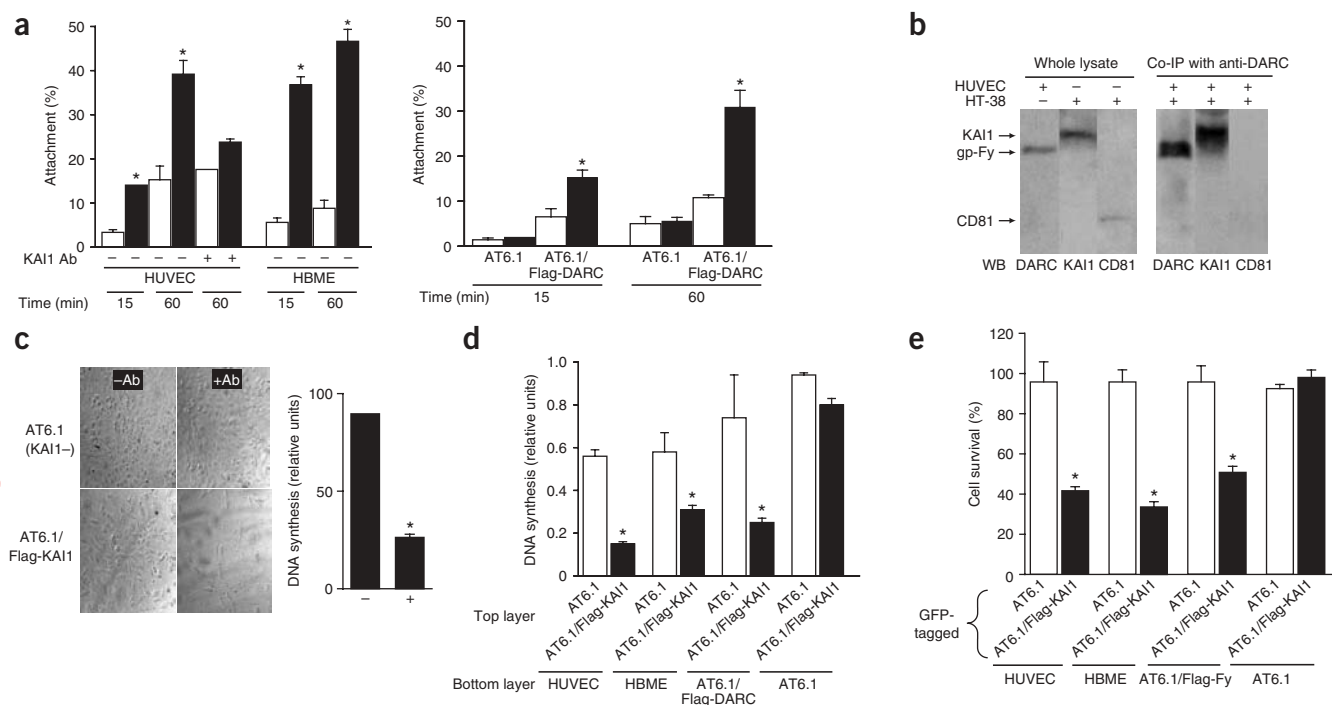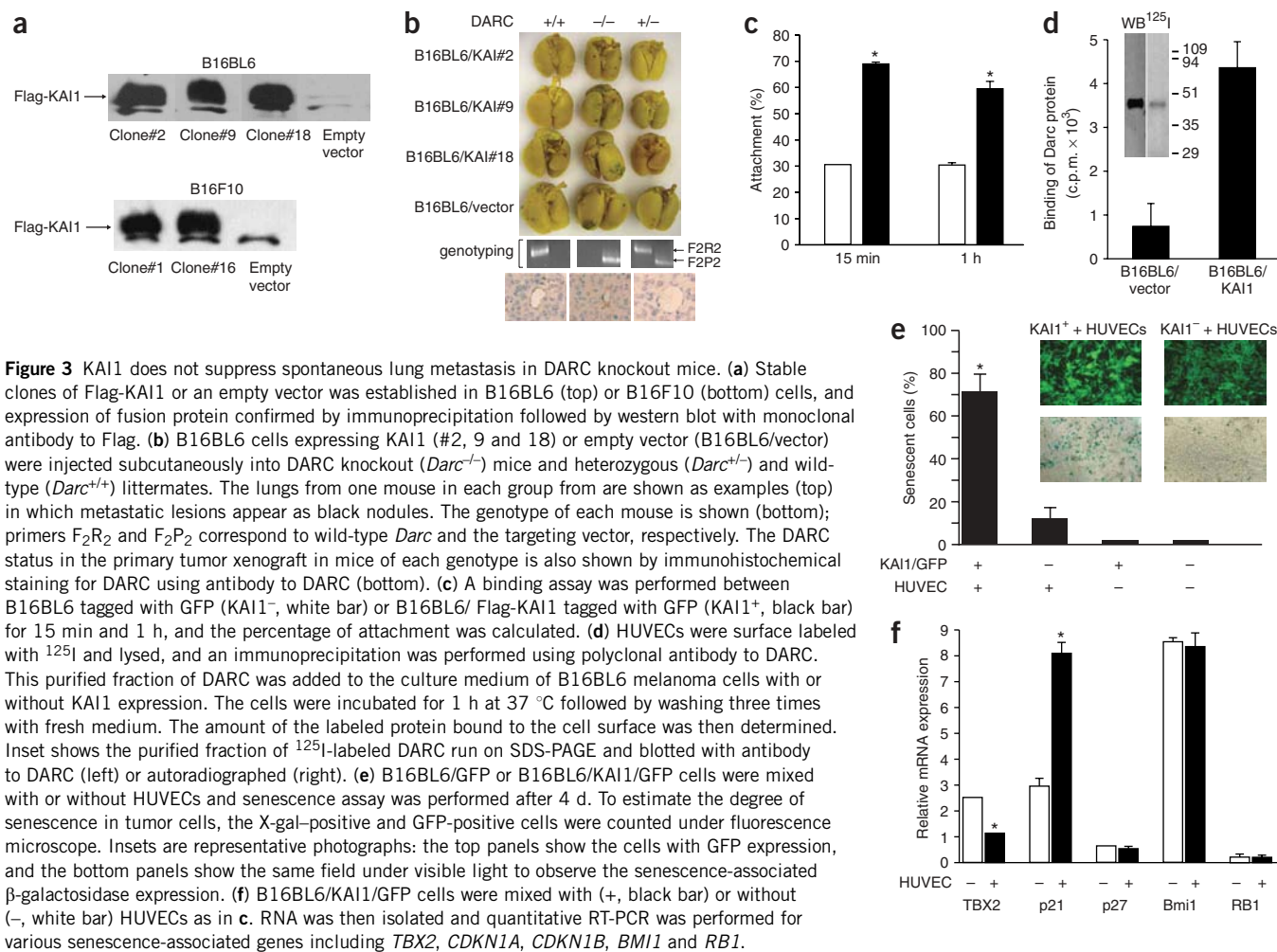

Figure 2 Interaction of KAI1 and DARC leads to growth arrest of cancer cells. (**a**) KAI1 selectively binds to cells expressing DARC in a cell-to-cell binding assay *in vitro*. HBMEs and HUVECs (DARC+; left), AT6.1 and AT6.1/Flag-DARC (right) were grown to confluency. Then, approximately 10³ cells of AT6.1 tagged with GFP (KAI1−, white bar) or AT6.1/Flag-KAI1 tagged with GFP (KAI1+, black bar) were added on the confluent cell layers, in the presence (+) or absence (−) of monoclonal antibody to KAI1 as indicated. After 15 min or 1 h, wells were washed and the percentage of attachment was calculated as described in Methods. (**b**) Endogenous KAI1 and DARC coimmunoprecipitate in mammalian cells. Lanes 1–3: expression level of KAI1 and CD81 in HT-38 cells and level of DARC in HUVECs were tested by western blot using antibodies to KAI1, CD81 and DARC, respectively. Lanes 4–6: HT-38 and HUVECs were mixed in the presence of a cell-impermeable crosslinker DTSSP for 30 min followed by immunoprecipitation with DARC antibody and western blot with antibodies to KAI1, DARC or CD81, as indicated. (**c**) Monoclonal antibody to KAI1 inhibits growth of KAI1+ prostate epithelial cells. AT6.1 (KAI1−) or AT6.1/Flag-KAI1 (KAI1+) were seeded and monoclonal antibody to KAI1 was added to the wells indicated by '+ Ab' and the rate of DNA synthesis was measured. (**d**) Suppression of DNA synthesis by DARC in prostate cancer cells. DARC+ endothelial cells (HUVECs, HBMEs) and cells with or without DARC expression (AT6.1, AT6.1/Flag-DARC; bottom layer) were grown to full confluency and incubated with 30 μM mitomycin C for 18 h. The cells were then washed extensively, and AT6.1 (white bars) or AT6.1/Flag-KAI1 (black bar) cells (top layer) were added on the monolayer, ³H-thymidine was added to the wells and the incorporation of radioisotopes into DNA of the attached cells was assayed. (**e**) Growth arrest in prostate cancer cells caused by interaction between KAI1 and DARC. Prostate cancer cells expressing both *CD82* and *GFP* genes (AT6.1/Flag-KAI1, black bars) or cells expressing only GFP (AT6.1, white bars) were mixed with cells with or without DARC expression for 1 h followed by plating in the presence of hygromycin, which allowed growth of only GFP-tagged AT6.1 or AT6.1/Flag-KAI1 cells. After 5 d, the number of colonies was counted under a fluorescent microscope. *$P < 0.05$.

Figure 3 KAI1 does not suppress spontaneous lung metastasis in DARC knockout mice. (**a**) Stable clones of Flag-KAI1 or an empty vector was established in B16BL6 (top) or B16F10 (bottom) cells, and expression of fusion protein confirmed by immunoprecipitation followed by western blot with monoclonal antibody to Flag. (**b**) B16BL6 cells expressing KAI1 (#2, 9 and 18) or empty vector (B16BL6/vector) were injected subcutaneously into DARC knockout ($Darc^{-/-}$) mice and heterozygous ($Darc^{+/-}$) and wild-type ($Darc^{+/+}$) littermates. The lungs from one mouse in each group from are shown as examples (top) in which metastatic lesions appear as black nodules. The genotype of each mouse is shown (bottom); primers F_2R_2 and F_2P_2 correspond to wild-type $Darc$ and the targeting vector, respectively. The DARC status in the primary tumor xenograft in mice of each genotype is also shown by immunohistochemical staining for DARC using antibody to DARC (bottom). (**c**) A binding assay was performed between B16BL6 tagged with GFP (KAI1$^-$, white bar) or B16BL6/ Flag-KAI1 tagged with GFP (KAI1$^+$, black bar) for 15 min and 1 h, and the percentage of attachment was calculated. (**d**) HUVECs were surface labeled with ^{125}I and lysed, and an immunoprecipitation was performed using polyclonal antibody to DARC. This purified fraction of DARC was added to the culture medium of B16BL6 melanoma cells with or without KAI1 expression. The cells were incubated for 1 h at 37 °C followed by washing three times with fresh medium. The amount of the labeled protein bound to the cell surface was then determined. Inset shows the purified fraction of ^{125}I-labeled DARC run on SDS-PAGE and blotted with antibody to DARC (left) or autoradiographed (right). (**e**) B16BL6/GFP or B16BL6/KAI1/GFP cells were mixed with or without HUVECs and senescence assay was performed after 4 d. To estimate the degree of senescence in tumor cells, the X-gal–positive and GFP-positive cells were counted under fluorescence microscope. Insets are representative photographs: the top panels show the cells with GFP expression, and the bottom panels show the same field under visible light to observe the senescence-associated β-galactosidase expression. (**f**) B16BL6/KAI1/GFP cells were mixed with (+, black bar) or without (−, white bar) HUVECs as in **c**. RNA was then isolated and quantitative RT-PCR was performed for various senescence-associated genes including *TBX2*, *CDKN1A*, *CDKN1B*, *BMI1* and *RB1*.

have a role in the metastasis-suppression action of KAI1. Rather, DARC seems to directly engage in the interaction with KAI1, which triggers an unknown signal pathway of growth arrest. To obtain mechanistic insight into the interaction between KAI1 and DARC that led to metastasis suppression in our *in vivo* model system, we first carried out a cell-to-cell binding assay using melanoma cells. We found that B16BL6 cells overexpressing KAI1 exhibited a significantly higher binding to the endothelial cells over different time points (**Fig. 3c**), which is consistent with our observation in the case of prostate tumor cells. We then tested the binding of ^{125}I-labeled purified fraction of DARC to the cell surface of B16BL6 melanoma cells with or without KAI1 expression. A significantly ($P < 0.05$) higher amount of DARC bound to the melanoma cells expressing KAI1 compared with the empty vector transfectant (**Fig. 3d**), supporting our notion that KAI1 and DARC interact at the surface of the tumor cells. Such interaction leads to growth arrest of tumor cells (**Fig. 2d,e**). However, we did not detect apoptosis in the KAI1$^+$ tumor cells by TUNEL assay upon coculturing with DARC$^+$ cells (data not shown). We therefore examined whether the interaction with DARC leads to senescence in the KAI1$^+$ tumor cells by mixing HUVECs with GFP-tagged B16BL6 cells with or without KAI1 expression. We found that a significant percentage of KAI1$^+$ tumor cells underwent senescence as a result of interaction with HUVECs (**Fig. 3e**). Furthermore, we found that expression of the senescence-associated gene *TBX2* was reduced and *CDKN1A* (encoding p21) was

upregulated in these cells upon interaction with HUVECs, whereas *CDKN1B* (encoding p27), *BMI1* or *RB1* did not show any appreciable change in expression level (**Fig. 3f**). Notably, several previous publications showed a potential link between tumor progression and senescence[14–18]. Particularly consistent with our results, TBX2 has been found to inhibit senescence by directly repressing p21 expression in melanoma cells, suggesting that the TBX2-p21 pathway has a crucial role in tumor progression[19].

Collectively, our results indicate that when tumor cells dislodge from the primary tumor and intravasate into the blood vessels, tumor cells expressing KAI1 attach to the endothelial cell surface, whereby KAI1 interacts with DARC. This interaction transmits a senescent signal to the tumor cells, whereas those that lost KAI1 expression proliferate in the circulation, potentially giving rise to metastases. Notably, KAI1 as a tetraspanin was previously shown to interact with several other cell-surface proteins including α4β1 integrin[20]. The presence of these integrins on tumor cells promotes attachment to vascular endothelial cells[21]. Therefore, the association of integrin and KAI1 may have a part in the KAI1-DARC interaction, although this possibility needs to be explored further. Nonetheless, our model of the mechanism of action of KAI1 explains how KAI1 suppresses metastasis without affecting formation of primary tumors. It highlights a previously unappreciated function of *DARC* and identifies DARC as a new candidate for potential therapeutic intervention for metastatic cancer.

Table 1 Spontaneous and experimental metastases of B16BL6/KAI1 cells in DARC knockout mice

Spontaneous metastases of B16BL6/KAI1 cells in DARC knockout mice

Clone #	KAI1 expression	Tumor volume (mean ± s.e.m.)			Incidence of pulmonary metastasis			
		$Darc^{+/+}$	$Darc^{-/-}$	$Darc^{+/-}$	$Darc^{+/+}$	$Darc^{-/-}$	$Darc^{+/-}$	P value
2	Positive	4.9 ± 0.03	4.5 ± 0.02	4.5 ± 0.01	2/15 (13.3%)	9/15 (60%)	1/15 (6.7%)	0.02[a], 0.008[b]
9	Positive	4.6 ± 0.05	4.5 ± 0.03	4.9 ± 0.04	1/15 (6.7%)	6/13 (46.2%)	1/15 (6.7%)	0.05[a], 0.05[b]
18	Positive	4.5 ± 0.05	4.2 ± 0.03	3.9 ± 0.04	0/13 (0%)	6/12 (50%)	0/13 (0%)	0.04[a], 0.04[b]
Empty vector	Negative	4.9 ± 0.05	4.8 ± 0.05	4.9 ± 0.03	6/15 (40%)	5/14 (35.7%)	5/14 (35.7%)	0.8[a], 0.89[b]

Experimental metastases of B16F10/KAI1 cells in DARC knockout mice

Clone #	KAI1 expression	Number of pulmonary metastases			
		$Darc^{+/+}$	$Darc^{-/-}$	$Darc^{+/-}$	P value
1	Positive	4.7 ± 2.4 ($n = 9$)	47.86 ± 5.9 ($n = 7$)	2.8 ± 0.8 ($n = 6$)	<0.001[a], <0.001[b]
16	Positive	4.4 ± 2.4 ($n = 7$)	32.14 ± 3.6 ($n = 7$)	9.4 ± 2.7 ($n = 5$)	<0.001[a], 0.001[b]
Empty vector	Negative	40.0 ± 8.4 ($n = 5$)	56.0 ± 11.8 ($n = 5$)	32.5 ± 4.8 ($n = 6$)	0.3[a], 0.08[b]

[a]Comparison between $Darc^{-/-}$ and $Darc^{+/+}$. [b]Comparison between $Darc^{-/-}$ and $Darc^{+/-}$.

METHODS

Yeast two-hybrid screening. We cloned full-length *KAI1* cDNA cloned into the yeast vector pEG202-NLS (Origene Technologies) as bait, and performed yeast two-hybrid screening and mating assay according to the manufacturer's protocol.

Quantitative β-galactosidase assay. We performed the β-galactosidase assay (Miller test) as previously described[22].

Cell culture. The rat prostatic carcinoma cell line AT6.1, the human breast carcinoma cell line MDA-MB-435, HBMEs and the mouse melanoma cell lines B16BL6 and B16F10 were provided by C. Rinker-Schaeffer (University of Chicago), B.E. Weissman (University of North Carolina at Chapel Hill), K. Pienta (University of Michigan Medical School) and I.J. Fidler (M.D. Anderson Cancer Center), respectively. We purchased the human lung epithelial carcinoma cell line A549 and colon carcinoma cell line HT-38 from American Type Tissue Culture Collection. We cultured the cells in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS, 250 nM dexamethasone and antibiotics. We obtained HUVECs from Clonetics and cultured them in endothelial growth medium (EGM, Clonetics) as per the manufacturer's instruction.

Immunoprecipitation and western blot. For coimmunoprecipitation experiments using the AT6.1 cells, approximately 48 h after transfection, we harvested cells and lysed them in ice-cold lysis buffer (1% NP40, 10 mM Tris, pH 8.0, 150 mM NaCl, 3 mM $MgCl_2$, 2 mM PMSF) for 45 min and centrifuged them at maximum speed for 15 min. For immunoprecipitation with monoclonal antibody to Flag, we used Flag-specific M2 affinity gel (Sigma). For immunoprecipitation with antibody to hemagglutinin, we incubated the lysate with monoclonal antibody to hemagglutinin (Boehringer Mannheim) and used protein G-Sepharose beads. After immunoprecipitation, we thoroughly washed the beads, and analyzed bound proteins by western blot using monoclonal antibody to hemagglutinin or monoclonal antibody to Flag (Sigma) at dilutions of 1:400 and 1:500, respectively. For coimmunoprecipitation of endogenous KAI1 and DARC, we mixed the KAI1^{+} tumor cell line HT-38 with DARC[+] HUVECs in the presence of the cell-impermeable cross-linker DTSSP for 30 min at 24 °C. We lysed the cells in the same lysis buffer as above, centrifuged them and immunoprecipitated the lysate with rabbit polyclonal antibody to DARC in the presence of protein G agarose beads. After immunoprecipitation, we analyzed bound proteins by western blot using antibody to DARC (1:500), mouse monoclonal antibody to KAI1 (1:1,000, a gift from O. Yoshie, Shionogi Institute for Medical Science) or mouse monoclonal antibody to CD81 (1:20, Chemicon).

Immunohistochemistry. We carried out immunohistochemical analysis on paraffin-embedded, surgically resected specimens of prostate, breast and lung, using polyclonal antibody to DARC. Briefly, we deparaffinized sections, rehydrated them and heated them at 80 °C for 20 min in 25 mM sodium citrate buffer (pH 9) for antigen exposure. We treated sections with 3% H_2O_2 to block endogenous peroxidase activity and then incubated them with primary antibody (1:50 dilution) for 1 h at 24 °C. After washing in Tris-buffered saline/0.1% Tween-20, we incubated sections with horseradish peroxidase–conjugated rabbit-specific IgG (Dako Corp.). We washed sections extensively, and applied DAB substrate chromogen solution followed by counterstaining with hematoxylin. The Southern Illinois University Institutional Review Board approved obtaining human specimens for this study.

Cell-to-cell binding assay. We seeded HBMEs, HUVECs, AT6.1 or AT6.1/Flag-DARC (DARC[+] permanent clone established in AT6.1) cells in 24-well plates and grew them to full confluency. We trypsinized cells used for overlaying (AT6.1/GFP and AT6.1/Flag-KAI1/GFP, or B16BL6/GFP and B16BL6/Flag-KAI1/GFP) and resuspended them in RPMI medium, and added 10^3 cells on the confluent bottom cell layers in the presence or absence of antibody to KAI1. After 15 min or 1 h, we washed the wells with RPMI medium three times and incubated the cells for 12 h at 37 °C. The numbers of cells attached on confluent monolayers were then counted by observing GFP signal under a confocal microscope and the percentage of attached cells was calculated. For each data point, experiments were performed in triplicate wells and ten random fields were counted in each well.

Treatment of tumor cells with monoclonal antibody to KAI1. We seeded approximately 10^3 cells of AT6.1 and AT6.1/Flag-KAI1 in 96-well plates. We then added ^{3}H-thymidine with or without monoclonal antibody to KAI1 (provided by H. Conjeaud, Cochin Hospital) to the wells, which we then incubated at 37 °C for 48 h. The ^{3}H-thymidine incorporation by the AT6.1/KAI1 cells was normalized with respect to the incorporation by the AT6.1 cells. Each experiment was performed in triplicate.

Measurement of DNA synthesis. We cultured HUVECs, HBMEs, AT6.1 and AT6.1/Flag-DARC cells to confluency and then treated them with mitomycin C for 18 h to block DNA synthesis. After washing the wells extensively with RPMI media, we seeded 10^3 AT6.1 cells that did or did not express KAI1 (AT6.1/Flag-KAI1 or AT6.1) on the monolayer of mitomycin C–treated cells and added ^{3}H-thymidine to the wells. We incubated the cells at 37 °C for 48 h, then washed the wells with RPMI media three times and measured the incorporation of ^{3}H-thymidine in the attached cells. The rate of DNA synthesis by the cells

seeded on monolayers was normalized by that of cells seeded directly on the plastic plate. Each experiment was performed in triplicate.

Colony formation assay. We trypsinized HUVECs, HBMEs, AT6.1 and AT6.1/DARC cells, resuspended them in RPMI medium and mixed them with AT6.1 cells, which expressed the gene encoding GFP with or without KAI1 (AT6.1 or AT6.1/Flag-KAI1, both GFP tagged), for 1 h, then plated the mixture in RPMI medium containing hygromycin. The GFP-tagged AT6.1 or AT6.1/Flag-KAI1 cells were also plated without mixing with HUVECs, HBMEs, AT6.1 or AT6.1/DARC cells for the purpose of normalization. We incubated the cells at 37 °C for 5 d and counted the number of colonies expressing GFP under the fluorescence microscope. The number of colonies formed by GFP-tagged AT6.1 or AT6.1/Flag-KAI1 mixed with HUVECs, HBMEs, AT6.1 and AT6.1/DARC cells was normalized with the number of colonies formed by the GFP-tagged cells alone. Each experiment was done in triplicate.

In vivo metastasis assay. For spontaneous metastasis assay, we injected approximately 0.5×10^6 cells/0.2 ml of PBS of various B16BL6 clones subcutaneously in the dorsal flank of the DARC knockout mice as well as heterozygous and wild-type littermates. We monitored mice daily for the growth of primary tumor. After 6 weeks, mice were killed, tumor volume was calculated using the equation Volume = (Width + Length)/2 × width × length × 0.5236, and metastatic lesions were counted macroscopically. For experimental metastasis assay, we injected approximately 0.5×10^6 cells/0.2 ml PBS of various B16F10 clones intravenously into the tail vein of the DARC knockout mice as well as control littermates. Mice were killed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically. All protocols were approved by the Southern Illinois University Institutional Review Board.

In vitro binding assay. The DARC$^+$ cells were surface labeled with ^{125}I using Iodo-beads (Pierce) according to the manufacturer's protocol. We lysed the cells and immunoprecipitated them using antibody to DARC and protein G agarose. We washed the agarose beads extensively to remove unbound proteins and eluted the bound proteins using 0.1 M glycine, pH 3.5, immediately followed by neutralization with 0.5 M Tris, HCl, pH 7.4, 1.5 M NaCl. This eluate was further concentrated by Centricon P10. B16BL6 cells with or without KAI1 expression were seeded in 24-well plates and grown to confluency. We added the purified protein to the cells in culture and 48 h later, washed the wells three times with fresh medium and determined the amount of the bound protein.

Senescence assay. We trypsinized B16BL6/GFP or B16BL6/Flag-KAI1/GFP cells, resuspended them in medium and mixed them with the DARC$^+$ HUVECs for 1 h followed by plating the mixture. We also plated the GFP-tagged cells without mixing with HUVECs as control. We incubated the cells at 37 °C for 4 d. We then performed a senescence assay using a senescence-associated β-galactosidase detection kit (Calbiochem) according to the manufacturer's instruction, and counted the X-gal–positive and GFP-positive cells under a fluorescence microscope.

Real-time RT-PCR. We mixed B16BL6/Flag-KAI1/GFP cells with or without the DARC$^+$ HUVECs for 1 h, and then plated the mixture and incubated it at 37 °C for 4 d. We isolated total RNA from the cells and reverse-transcribed it. We then amplified the cDNA with a pair of mouse-specific forward and reverse primers for the following genes: TBX2 (forward, 5′-CACCTTCCGCACCTAT GTC-3′; reverse, 5′-CAAACGGAGAGTGGGCAGCGTT-3′), CDKN1A (forward, 5′-CCGTGGACAGTGAGCAGTT-3′; reverse, 5′-CCAATCTGCGCTTG GAGTGA-3′), BMI1 (forward, 5′-AATCCCCACTTAATGTGTGTC-3′; reverse, 5′-TCACCTCTTCCTTAGGCTTCTC-3′), CDKN1B (forward, 5′-GTGGAC CAAATGCCTGACT-3′; reverse, 5′-GGCGTCTGCTCCACAGTG-3′), RB1 (forward, 5′-TGATGAAGAGGCAAACGTGG-3′; reverse, 5′-TGGCCCACAGCG TTAGCAAAC-3′) and β-actin. We performed PCR using DNA engine opticon2 system (MJ Research) and the Dynamo SYBR Green qPCR Kit (Finnzyme

Corp). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 15 min followed by 30 cycles of PCR using the following profile: 94 °C for 30 s; 57 °C for 30 s; 72 °C for 30 s.

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Interaction of Duffy Antigen Receptor for Chemokines and KAI1: A Critical Step in Metastasis Suppression

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Abstract

Tumor metastases suppressor protein KAI1/CD82 is capable of blocking the tumor metastases without affecting the primary tumor formation, and its expression is significantly down-regulated in many types of human cancers. However, the exact molecular mechanism of the suppressor function of KAI1 remains elusive. Evidence from our laboratory supports a model in which tumor cells dislodge from the primary tumor and intravasate into the blood or lymphatic vessels followed by attachment to the endothelial cell surface whereby KAI1 interacts with the Duffy antigen receptor for chemokines (DARC) protein. This interaction transmits a senescent signal to cancer cells expressing KAI1, whereas cells that lost KAI1 expression can proliferate, potentially giving rise to metastases. Our model of the mechanism of action of KAI1 shows that metastasis suppressor activity can be dependent on interaction with host tissue and explains how KAI1 suppresses metastasis without affecting primary tumor formation. Taken together, *in vitro* and *in vivo* studies identify the KAI1-DARC interaction as a potential target for cancer therapy. [Cancer Res 2007;67(4):1411–4]

KAI1 Blocks Metastases without Affecting Primary Tumor Formation

When cancer is diagnosed, the most critical question is whether the disease is localized or has it already disseminated to other parts of the body. Unfortunately, the majority of patients already have a clinically undetectable metastatic disease at the time of a visit to the clinic, and >90% of cancer patients ultimately succumb to sequelae of metastatic disease. Following primary tumor formation, a population of tumor cells can acquire molecular and cellular changes, which enable cancer to spread to distant sites. These include invasive phenotype that results in the loss of cell-cell adhesion and cell-extracellular matrix adhesion followed by proteolytic degradation of the matrix. Additional changes are needed in order for cells to intravasate into neighboring blood and lymphatic vessels and disseminate through the circulation. Those cells that survive in the circulation are arrested at distant organ sites, extravasate, and lodge at the secondary sites, where the cells must also proliferate and colonize for successful metastasis. The molecular mechanism(s) regulating acquisition of metastatic ability remains poorly understood despite the urgent need for development of novel treatment options for patients with metastatic disease. The discovery of a series of metastasis suppressor genes in the past decade has shed new light on many crucial aspects of this intricate biological process. The metastases suppressor genes and their encoded proteins, by definition, suppress the process of metastasis without affecting tumorigenesis. To date, more than a dozen of these genes have been identified and include *nm23, KAI1, Kiss1, BRMS1, MKK4, RhoGDI2, RKIP, Drg-1, CRSP3, SSeCKs, TXNIP/VDUP-1, Claudin-4*, and *RRM1* (1).

The *KAI1* gene was originally isolated as a prostate-specific tumor metastasis suppressor gene using the microcell-mediated chromosome transfer method followed by Alu-PCR. It is located in the p11.2 region of human chromosome 11 (2, 3). When the *KAI1* gene was transferred into highly metastatic Dunning rat prostatic cancer cells, KAI1-expressing cancer cells were suppressed in their metastatic ability in mice, whereas their primary tumor growth was not affected (2). The DNA sequencing analysis of the *KAI1* gene revealed that it is identical to *CD82*, a surface glycoprotein of leukocytes (3). The protein has four hydrophobic and presumably transmembrane domains, two extracellular domains, and three short intracellular domains and belongs to the family of tetraspanins. Immunohistochemical analysis of human prostate tumor samples revealed that the KAI1 expression was down-regulated in >70% of the primary tumors (4). Similar results were also observed in other types of tumors, including lung, breast, pancreatic, colon, bladder, ovarian, hepatocellular carcinoma, and melanoma (1, 5). *KAI1* gene expression is correlated with poor survival in patients with these types of cancers. Therefore, the KAI1 is a bona fide metastasis suppressor protein in multiple cancer types. This raises the intriguing question of how the *KAI1* gene suppresses the metastasis process.

Duffy Antigen Receptor for Chemokines on Endothelial Cell Plays a Key Role in the Suppressor Function of KAI1

To understand the mechanism of KAI1 in metastasis suppression, a yeast two-hybrid system was used to systemically screen interacting proteins of KAI1 and found that KAI1 was physically associated with the Duffy antigen receptor for chemokines (DARC; ref. 6). DARC is a promiscuous CXC chemokine receptor that is strongly expressed on the endothelial cells of lymphatic and blood vessels as well as on RBCs. Immunohistochemical analyses confirmed that DARC is highly expressed in the endothelium, particularly in the small veins and venules as well as lymphatic vessels in both normal and tumor tissue of the prostate and breast, whereas its expression is undetectable in the epithelial cells or stroma. On the other hand, KAI1 is highly expressed in the normal epithelial cells in these organs, and the expression is significantly reduced in carcinoma. Based on this spatial localization, we hypothesized that KAI1 on epithelial cells interacts with the DARC when cancer cells expressing KAI1 intravasate and encounter the endothelial lining of small blood vessels. A series of experiments

done to test this putative interaction using human endothelial cells found that tumor cells expressing KAI1 indeed bound to endothelial cells and this binding was blocked by KAI1 antibody. These results prompted the question "what is the physiologic outcome of the interaction between KAI1 expression tumor cells and DARC on endothelial cells?" Results from recent studies on T-cell activation provided clues crucial to answering this question. KAI1/CD82 is barely detectable on resting peripheral T and B lymphocytes, whereas its expression is highly up-regulated on activation of these cells. This up-regulation is associated with morphologic changes and expression of activation markers, such as CD82 and MHC II antigens. Lebel-Binay et al. described that the coengagement of KAI1/CD82 and T-cell receptor by anti-CD82 monoclonal antibody (mAb) and anti-CD3 mAb, respectively, was able to activate T cells *in vitro*. Specifically, when T cells are stimulated *in vitro* by anti-KAI1/CD82 mAb, KAI1/CD82 seems to transmit a signal that results in tyrosine phosphorylation, a rapid increase in intracellular Ca^{2+} level, and interleukin-2 production (7). Interestingly, this activation was associated with a change in cellular morphology and inhibition of cell proliferation (8). Therefore, we hypothesized that engagement of KAI1/CD82 on cancer cells may also activate a similar signal pathway, which results in growth arrest of tumor cells. Consistent with this hypothesis, the addition of anti-CD82 antibody in the culture of KAI+ cells resulted in significant growth suppression of tumor cells,

which was also observed when the tumor cell was cocultured with human endothelial cells. Therefore, our data strongly suggest that growth suppression is determined by dynamic and reciprocal interaction of KAI1 on cancer cells and DARC on endothelial cells in the vasculature (Fig. 1).

To further corroborate these findings, melanoma cells with or without expression of KAI1 were transplanted into DARC knockout as well as wild-type mice and resultant overt lung metastases were quantitated. Primary tumors developed in all mice without significant changes of growth rate regardless of the KAI1 level in the tumor cells and DARC status of the mice. However, the KAI1-positive cell lines developed significant number of pulmonary metastases in the DARC knockout mice, whereas metastasis was almost completely abrogated when the same cell lines were injected into the heterozygote and wild-type littermates. Thus, in the absence of DARC, tumor cells expressing high level of KAI1 formed metastases, supporting our model that the metastasis suppressor function of KAI1 is dependent on the interaction of KAI1 and DARC on endothelial cells. The biochemical nature of this growth suppression through the KAI1-DARC interaction is of significant interest. To explore this, green fluorescent protein–labeled tumor cells were cocultured with endothelial cells and found that KAI1-positive cells became senescent without a sign of apoptosis. Further, senescence was associated with down-regulation of
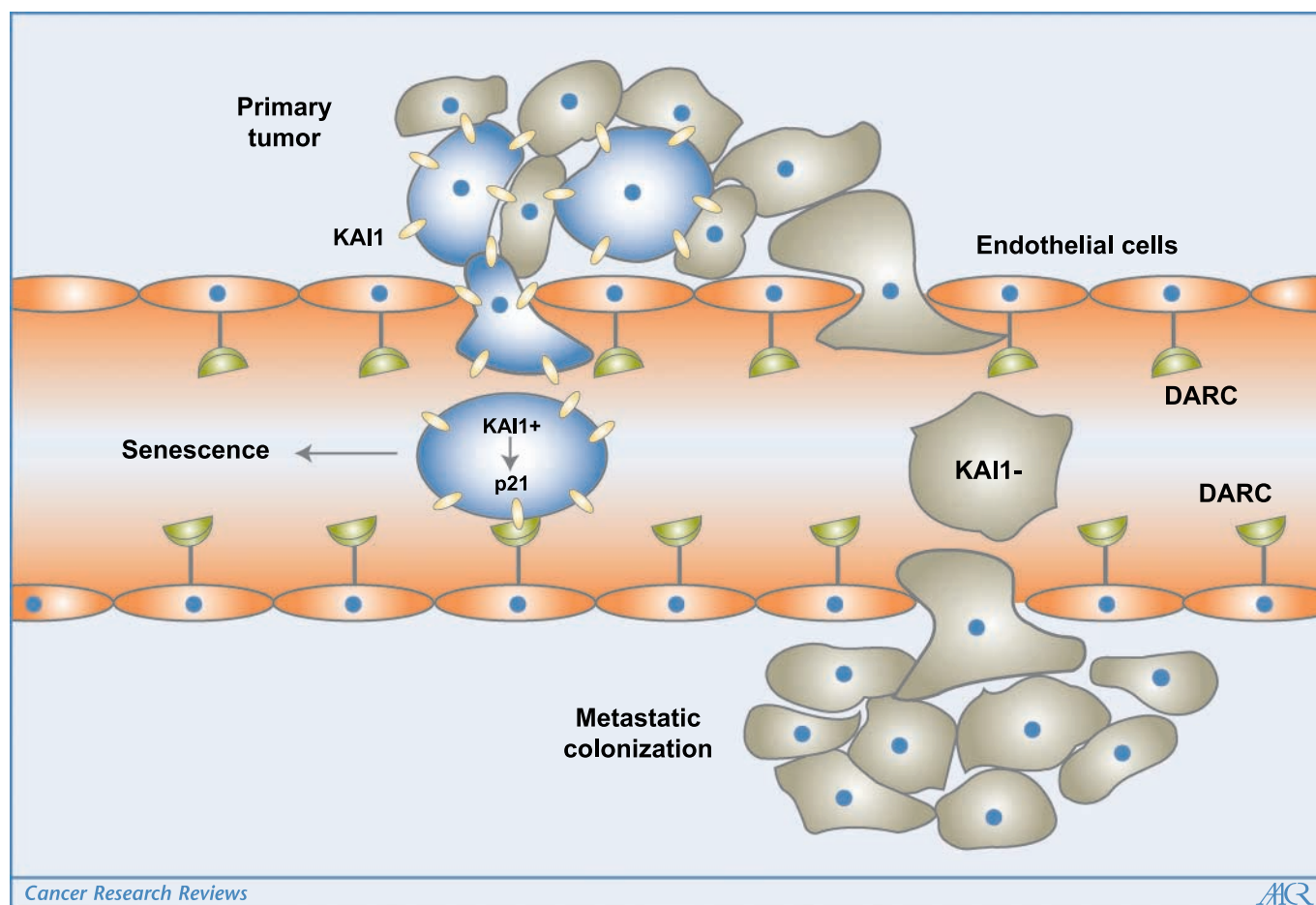

Figure 1. KAI1-DARC interaction blocks tumor metastasis.

TBX2 and up-regulation of the cyclin-dependent kinase inhibitor p21. These studies suggest that growth suppression induced by the KAI1-DARC interaction is due to the activation of p21 followed by cellular senescence.

DARC Signal for Cancer Therapy?

Our model of the mechanism of action of KAI1 explains how KAI1 suppresses metastasis without affecting primary tumor formation. However, it has also provoked many critical questions about (*a*) whether KAI1 function requires other "cofactors," (*b*) what cellular signal is induced by DARC and KAI1 interaction, and (*c*) whether KAI1-DARC signal be targeted for cancer therapy.

KAI1 has been reported to be associated with many different membrane proteins, including integrins, Kitenin, epidermal growth factor receptor (EGFR), CD63, CD9, EW12, and c-Met (9–14). KAI1 belongs to the transmembrane 4 superfamily, which is known to form a multiprotein complex referred to as tetraspanin web (9) that also interacts with integrins. KAI1 was indeed found to be associated with various integrins, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, and the complex of integrin $\alpha_3\beta_1$ and KAI1 was reported to suppress fibronectin/$\alpha_3\beta_1$-induced cell invasion through inhibition of the cytoskeletal system (10). KAI1 was also found to be associated with other tetraspanins, including CD9 and CD63 (11). The functions of these molecules are not well understood; however, the loss of expression of CD9 and CD63 correlates with poor prognosis and increased metastasis (12). Therefore, these two tetraspanins may also play an important role in the KAI1 function. More recently, Lee et al. (13) found that KAI1 is associated with another tetraspanin molecule, Kitenin, whose overexpression promoted increased tumorigenicity and metastasis *in vivo*. The exact molecular function of Kitenin is yet to be understood; however, it was proposed that Kitenin decreases the metastasis suppressor functions of KAI1 and/or cytoplasmic signaling pathway that shifts the invasive/anti-invasive balance toward invasion. In addition to integrins and tetraspanins, Odintsova et al. (14) recently found that KAI1 physically associates with the EGFR and rapidly desensitizes the EGF-induced signal, which could lead to suppression of cell migration, although it is as yet unclear whether this mechanism indeed accounts for the metastasis suppression *in vivo*. Nevertheless, KAI1 seems to be able to interact with various proteins on the membrane. It is yet to be determined whether all these components indeed form a "multiprotein complex" and whether they are all necessary or sufficient for the metastasis suppressor function of KAI1 because these proteins associated with KAI1 have been identified in different systems.

DARC-KAI1 interaction seems to transduce cytoplasmic signal to nucleus to modulate TBX2 and p21 expression and induce senescence. The signal transduction mechanism involved in this senescence pathway is a crucial question. Thus far, however, little information is available about the signals related to the KAI1 function. Zhang et al. (10) recently reported that protein kinase C (PKC) is associated with various tetraspanins, including KAI1, and that these tetraspanins act as linker molecules to recruit PKC into proximity with specific integrins. It was also shown that only those integrins ($\alpha_3\beta_1$ and $\alpha_6\beta_1$) that strongly associated with tetraspanins, such as KAI1, were in association with PKC. Therefore, KAI1 may act as a modulator of the PKC signal, which plays a crucial role in cell cycle progression, migration, and invasiveness as well as in cell cycle arrest (15, 16). Interestingly, PKC was found to be able to directly modulate p21 expression and activity (15), which is the hallmark of KAI1/DARC-induced senescence. More recently, KAI1 has been shown to suppress integrin-dependent activation of the receptor kinase c-Met (17). The ligand of c-Met is hepatocyte growth factor, which is capable of both stimulating and arresting cell cycle, and these effects in either case are mediated through the up-regulation or down-regulation of p21. Therefore, PKC and c-Met pathway may play key roles in the KAI1/DARC-induced signaling, although this possibility needs to be tested directly.

DARC is the receptor of the malaria parasite *Plasmodium vivax*. Approximately 70% of West African descendants have lack of expression of DARC on erythrocytes, thereby resistant to malaria infection. Interestingly, the same population showed significantly higher incidence of both prostate and breast cancer as well as higher rate of metastatic disease than white (18). DARC also serves as promiscuous receptor for both C-C and CXC chemokines, which is believed to function as "decoy" of excess chemokines. Therefore, DARC is proposed to play a role as antimetastatic molecule by clearing angiogenic CXC chemokines. In fact, Shen et al. (19) recently have shown that growth of prostate tumor was significantly augmented in DARC-deficient mice compared with the wild-type. More recently, it was also reported that overexpression of DARC in breast cancer cells significantly suppressed the spontaneous pulmonary metastasis (20). Therefore, DARC may function as a metastases suppressor by two different mechanisms (i.e., by inducing KAI1 signal in tumor cells and by sequestering angiogenic factors). This raises an attractive possibility of developing antimetastatic drugs that mimic the action of DARC. Further understanding of the biochemistry of the interaction of KAI1-DARC and their cofactors as well as the downstream signal should lead to effective therapeutic strategy against metastatic cancer.

Acknowledgments

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Tumor–endothelial cell interactions: Therapeutic potential

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Abstract

Metastasis is the primary cause of death in cancer patients. However, the molecular mechanism of the metastatic process is poorly understood because it involves multiple steps with a high degree of complexity. A critical step for successful establishment of secondary colonization is the hematogenous dissemination of malignant cells. During this process, the attachment of cancer cells to the endothelial cells on microvasculature is considered to be an essential step and many adhesion molecules as well as chemokines have been found to be involved in this process. This interaction of cancer–endothelial cell is considered not only to determine the physical site of metastasis, but also to provide the necessary anchorage to facilitate tumor cell extravasation. However, recent evidence indicates that this interaction also serves as a host defense mechanism and hinders the process of metastasis. The tumor metastases suppressor gene, KAI1, has been known to block metastatic process without affecting the primary tumor growth, and this protein has been found to be able to bind to the chemokine receptor, Duffy antigen receptor for chemokines (DARC), which is expressed on endothelial cells. Importantly, this interaction markedly induces senescence of tumor cells. This novel finding is not only significant in the context of molecular dissection of metastatic process but also in the therapeutic implication to develop drugs inhibiting metastasis.
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Keywords: Endothelial cell; Metastasis suppressor; KAI1; Senescence; DARC

Contents

Metastatic disease is the major cause of cancer death

After decades of extensive effort and investment in cancer research, we are finally observing a declining trend in cancer death, which is mainly attributed to early detection and preventive measures for various types of cancer.

However, cancer still accounts for the second leading cause of death, and more than half a million people succumb to the disease every year in the USA alone (American Cancer Society, 2006). At the time of diagnosis of cancer, the most critical question for the treatment is whether the tumor is localized or has it already metastasized. Unfortunately, there are no effective

treatment options available for patients with metastatic cancer, and more than 90% of patients ultimately die due to sequelae of metastatic disease. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the field of cancer research. This is attributed to the fact that metastasis involves multiple intricate steps with a high degree of complexity.

The first step in metastasis is the detachment of tumor cell from primary tumor mass. A major factor that enables tumor cells to become motile is decreased adhesiveness which is mediated by hydrolyses originating from the central necrotic area of large tumors (Sylven, 1973). In addition, certain cellular factors such as the autocrine motility factor (AMF) (Stracke et al., 1987) and hepatocyte growth factor (HGF) (Michalopoulos, 1990) are also found to be active players in this process. The tumor cell detachment from the primary tumor mass is followed by intravasation during which neoplastic cells must traverse barriers of collagen and elastic structures in the interstitial tissues and basement membrane, a hallmark of invasion front in all forms of cancer (Beitz and Calabresi, 1993). Enzymes capable of degrading the extracellular matrix are important in this process. A positive association with tumor aggressiveness has been reported for various classes of degradative enzymes including serine-, thiol-proteinases (Reich et al., 1988; Recklies et al., 1982; Sloane and Honn, 1984), heparanases and metalloproteinases such as matrix metallopeptidase 2 (MMP2) and matrix metallopeptidase 9 (MMP9) (Wang et al., 1980). Protease secretion by invading cells is often coupled to changes in cell shape and locomotion (Kalebic et al., 1983). The regulation of proteolysis can take place at many levels because proteinase inhibitors can be produced by the host and by the tumor cells themselves (Murphy et al., 1981). Such proteinase inhibitor proteins including tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitors (PAIs) are considered to act as metastasis suppressor proteins although there are some controversies about the role of PAIs in metastases. Once a neoplastic cell has invaded the host circulatory system, it must survive in this hostile environment that includes mechanical damage, lack of growth factor from the original environment and the host immune system (Nicolson and Poste, 1983). Tumor cells that have survived and reached the endothelium of a distant organ extravasate using the same hydrolytic enzymes used for the initial step of invasion and form a secondary metastatic tumor. Therefore, tumor cells must undergo complex steps that involve many factors for successful metastasis.

Metastatic tumor cell interacts with the endothelium in vascular system

When the primary tumor grows more than 1 mm^3 in size, it can no longer obtain oxygen and nutrients by diffusion and hence initiates the process of angiogenesis. Therefore, many types of cancers secrete angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) and stimulate the vascular formation around the tumor

(Folkman, 1990; Ferrara, 2002). The tumor vasculature appears to have distinct features compared to the normal vessels in its structure and cellular components. The majority of tumor vessels have abnormal branching pattern with thin and leaky walls (McDonald and Baluk, 2002; Maniotis et al., 1999; Folberg et al., 2000; Hashizume et al., 2000). However, a functional vessel-network is essential not only for providing nutrients for tumor growth but also for metastatic spread of tumor cells to distant organs (Yano et al., 2003). In general, cancer cells acquire their invasive property at a relatively early stage, during which many tumor cells are constantly shed into the circulatory system in patients (Hashizume et al., 2000; Daldrup et al., 1998; Ellis and Fidler, 1996). However, considering the number of tumor cells in the circulation, an incidence of successful metastasis to distant organs is a very rare event. This is mostly due to the body's immune system including cytotoxic T-lymphocyte (CTL) and innate immune cells that effectively eliminate the circulating tumor cells. Cancer cells, on the other hand, utilize a number of strategies to evade the immune surveillance, such as down-regulation of major histocompatibility complex (MHC) and tumor-specific antigens and formation of tumor-cell–platelet aggregates (Sadelain et al., 2003; Nieswandt et al., 1999).

The tumor cells that survive the harsh environment and finally reach the distant organs still need to extravasate to establish secondary colonization. How the tumor cells initiate this process is a critical question and it is yet to be clarified. However, two possible mechanisms have been proposed. One is tumor cell arrest at the capillaries (Chambers et al., 2002). Tumor cells often aggregate with platelets, and due to the size of their mass they are physically "trapped" in the capillaries of the distant organs. In fact, using fluorescence-tagged tumor cell and a video-capturing image technique, Weiss et al. found that many tumor cells injected into mice intraperitoneally were indeed "arrested" in capillaries (Weiss et al., 1992). These "arrested" tumor cells either remain dormant at the site or start growing and eventually extravasate by rupturing the blood vessel or by inducing the secretion of proteolytic enzymes. However, the majority of cancer cells that are "arrested" in capillaries appear to die due to deformation and surface-membrane rupture (Weiss et al., 1992). Therefore, this process may not be an efficient strategy for tumor cells to establish metastatic colonization at a distant site.

The other proposed mechanism of extravasation of tumor cells mimics the infiltration of leukocytes to the inflammatory site, and this process requires the adhesive property of tumor cells to bind to the endothelial cells of blood vessels. When leukocytes are attracted to the site of inflammation via chemokine gradient, their extravasation occurs in four steps (Dunon et al., 1996; Goodman et al., 2003). The first step is "rolling and adhesion" involving selectins which is a reversible process. Leukocytes adhere to the vessel wall via selectin molecules and "roll" along the endothelium. The second step is "tight binding" of leukocytes to the endothelium through other adhesion molecules on the endothelial cells such as integrins, intercellular adhesion molecule 1 (CD54), human rhinovirus receptor (ICAM1) and platelet/endothelial cell adhesion

molecule (CD31 antigen) (PECAM1). The third step is "diapedesis" and the leukocytes start extravasating or crossing the endothelial wall. This step also involves adhesion molecules such as leukocyte functional antigen-1 (LFA-1) and macrophage receptor 1 (MAC-1) and this interaction enables the leukocytes to migrate through the endothelial cell junction or squeeze their cell bodies through narrow pores (Engelhardt and Wolburg, 2004). The final step is the migration of leukocytes to the inflammatory site by various types of chemokines including interleukin 8 (IL8). Because many cancer cells express similar adhesion molecules that are also expressed on the migrating leukocytes, it is thought that cancer cells use a similar strategy for adhesion to the endothelial cells during metastasis. Initial studies to demonstrate the adhesive property of cancer cell to endothelial cells were done in tissue culture systems which were essentially static systems devoid of shear forces associated with physiological blood flow. In this *in vitro* system the cancer cells were added to monolayers of endothelial cells and the number of adhered cells was quantified in the presence and absence of various factors. It was found that there was a marked increase in adhesion of cancer cells to endothelial cells in the presence of cytokines such as interleukin-1 beta (IL-1β) (Okada et al., 1994), interleukin 1 (IL-1) (Lafrenie et al., 1994) and tumor necrosis factor alpha (TNF-α) (Okada et al., 1994; Sheski et al., 1999) that are known to induce the expression of adhesion molecules. Hence, these data serve as important evidence that cancer cells indeed bind to the endothelial cells. This interaction of cancer–endothelial cells is mediated by various adhesion molecules expressed on both cancer and endothelial cells. E-selectin which is expressed on endothelial cells binds to its ligand sialyl Lewis-X (SLE) or A-antigen expressed on cancer cells of colon and renal cell carcinoma (Lafrenie et al., 1994; Steinbach et al., 1996; Tozawa et al., 1995; Ye et al., 1995). Among the immunoglobulin superfamily, vascular cell adhesion molecule 1 (VCAM-1) expressed on endothelial cells was also found to bind to α4 integrins expressed on renal cell carcinoma (Lafrenie et al., 1994; Steinbach et al., 1996; Taichman et al., 1991; Tomita et al., 1995) and α4β1 expressed on melanoma (Garofalo et al., 1995) and sarcoma cells (Paavonen et al., 1994). The integrin adhesion molecules like α6 on endothelial cell mediate the adhesion of α6β1 on highly metastatic B16/129 melanoma cells to the luminal surface (Ruiz et al., 1993). In another similar instance, αvβ3 on endothelial cells have also been shown to bind to small cell carcinoma cells (Sheski et al., 1999). A more recent evidence shows that the expression of αvβ3 integrin (CD51/CD61) on human melanoma cells is required for adhesion of cancer cells with human Thy-1 cell surface antigen (THY1) expressed on the activated endothelium. Thus various molecules expressed on endothelial cells ranging from selectins, integrins and immunoglobulins superfamily are necessary for adhesion to the cancer cells for their emigration and subsequent formation of metastatic tumors. To mimic the cancer–endothelial cell interaction under a physiological condition of blood flow which generates shear force, the static system was modified by introducing fluid flow which can simulate the physical properties of microvascular blood flow. By using this system, E-selectin-dependent 'rolling'

has been observed in colon, ovarian, breast and melanoma cancer cells under dynamic flow condition (Brenner et al., 1995; Giavazzi et al., 1993; Tozeren et al., 1995). To further validate cancer–endothelial cell interaction in living organisms, Al-Mehdi et al. recently used the intravital videomicroscopy technique, generally used to observe adhesion of circulating intravascular cells, and indeed detected metastatic tumor cells attached to the endothelium of pre-capillary arterioles and capillaries in intact mouse lungs (Al-Mehdi et al., 2000). They injected Green Fluorescent Protein (GFP)-tagged metastatic cells into a mouse and traced the behavior of these tumor cells and found that the majority of the cells were attached to the endothelium of pre-capillary arterioles although rolling-like movement was not observed during that time period. Therefore, they concluded that tumor cells were not "arrested" at the capillaries, rather they preferentially attached to the endothelium before proceeding towards extravasation. They also found that tumor cells attached to the endothelium proliferate before extravasation. Therefore, this "attachment" of tumor cells to the endothelium appears to be an advantage for the tumor cells because it not only determines the physical site of metastasis, but also provides the necessary anchorage that prevents anoikis and facilitates cell proliferation.

Tumor metastases suppressor, KAI1, interacts with DARC on endothelial cells

The attachment of tumor cells to the endothelium appears to provide a platform for tumor cells to extravasate and proliferate for successful metastases. However, recent evidence indicates that this is not always the case and that the interaction of tumor–endothelial cells is rather a host defense mechanism to block metastasis process when tumor cells possess the metastases suppressor protein, KAI1, on the cell surface (Bandyopadhyay et al., 2006). The KAI1 gene was originally isolated as a prostate-specific tumor metastasis suppressor gene, using the microcell-mediated chromosome transfer method (Dong et al., 1995). The KAI1 gene is identical to CD82 which is located in the p11.2 region of human chromosome 11 (Dong et al., 1997). The protein has four hydrophobic and presumably transmembrane domains, two extracellular domains and three short intracellular domains (Engel and Tedder, 1994). When the KAI1 gene is transferred into a highly metastatic cell, metastatic ability of KAI1-expressing cell is suppressed in mice, whereas their primary tumor growth is not affected (Dong et al., 1995). Consistent with the *in vitro* and *in vivo* results, the immuno-histochemical analysis of various types of human cancers revealed that KAI1 expression is significantly reduced in advanced and metastatic tumors (Rinker-Schaeffer et al., 2006; Furuta et al., 2006).

To elucidate the molecular mechanisms of metastases suppression by the KAI1 gene, Bandyopadhyay et al. recently utilized the yeast two hybrid system to screen interacting proteins of KAI1 and found that the KAI1 protein is capable of binding to the promiscuous chemokine (C-X-C motif) (CXC) receptor, DARC, which is highly expressed on the surface of endothelial cells of lymphoid and blood vessels, particularly in

small veins and venules (Bandyopadhyay et al., 2006). Immunohistochemical analyses indeed indicate that the pattern of expression of DARC and KAI1 is opposite in endothelium and cancer cells. KAI1 is highly expressed in normal epithelial cells but undetectable in endothelial cells, while DARC is strongly expressed in the endothelium and not in epithelial cells (Bandyopadhyay et al., 2006). The results of a series of experiments including *in vitro* binding assay and co-immunoprecipitation as well as antibody inhibition assay demonstrated that KAI1 indeed binds to DARC on endothelial cells (Bandyopadhyay et al., 2006). Therefore, it is reasoned that KAI1 on cancer cells interacts with DARC only when they intravaste into the blood vessel. To test this hypothesis, tumor cells ectopically expressing the KAI1 gene were injected into wild type or DARC knockout mice subcutaneously or intravenously, and the incidence of lung metastases was examined (Bandyopadhyay et al., 2006). The result of this *in vivo* experiment was striking. The number of pulmonary metastases was significantly increased with the KAI1+ cells that were injected into the DARC knockout mice compared to the wild type animals. These results strongly support the notion that the metastasis suppressor function of KAI1 is dependent upon the interaction of KAI1 and DARC on endothelial cells. Their results also suggest that the inefficiency of metastasis through the "capillary arrest" is partly due to the trapping of tumor cells by endothelium via KAI1–DARC interaction.

The physiological relevance of the KAI1–DARC interaction is of paramount interest. Crucial clue to answer this question came from the recent study of T-cell activation. When T and B cells are activated, KAI1 (also called CD82) is significantly up-regulated while it is undetectable at resting stage. Interestingly, co-engagement of KAI1/CD82 and T-cell receptor (TCR) by anti-KAI1 mAb and anti-CD3 mAb, respectively, was able to activate T cells *in vitro*. When KAI1/CD82 is bound to the antibody, KAI1 appears to transmit an intracellular signal to

phosphorylate tyrosine followed by rapid increase in intracellular Ca2+ level and interleukin-2 (IL-2) production (Lebel-Binay et al., 1995). Importantly, this activation triggered changes in cell morphology and inhibition of cell proliferation (Lagaudriere-Gesbert et al., 1998). To test whether the activated KAI1 signal can also block the cell proliferation of epithelial tumor cells, the effect of the same antibody on the KAI1+ tumor cells was examined (Bandyopadhyay et al., 2006). The results of this experiment revealed that the addition of anti-KAI1 antibody to the culture of KAI1+ cells significantly suppressed the growth of the tumor cells, and the same effect was observed when KAI1+ cells were co-cultured with endothelial cells. These results strongly suggest that the activation of KAI1 via antibody or DARC triggers a growth inhibitory signal of tumor cells. The next question is as to what signals and genes are responsible for the growth inhibition. To explore this question further, GFP-labeled tumor cells with or without expressing KAI1 were co-cultured with human endothelial cells and the degree of apoptosis and senescence was examined. The results clearly indicated that the growth inhibitory effect by the KAI–DARC interaction was not due to apoptosis but senescence and that the induction of senescence was associated with the up-regulation of senescence-controlling genes, T-box 2 (TBX2) and p21 genes (Bandyopadhyay et al., 2006). Therefore, these *in vitro* and *in vivo* results are consistent with the notion that KAI1 suppresses metastases by interacting with DARC on endothelial cells after intravasation followed by induction of cell senescence and that this senescence is triggered by activation of TBX2 and p21 (Fig. 1). The tumor cells at the senescent stage are expected to be cleared swiftly by immune cells in the blood vessels as Xue et al. recently reported that senescent tumor cells trigger innate immune responses which target these tumor cells and that this mechanism was dependent on transient expression of the p53 gene in tumor cells (Xue et al., 2007). In this context, it is noteworthy that the expression of
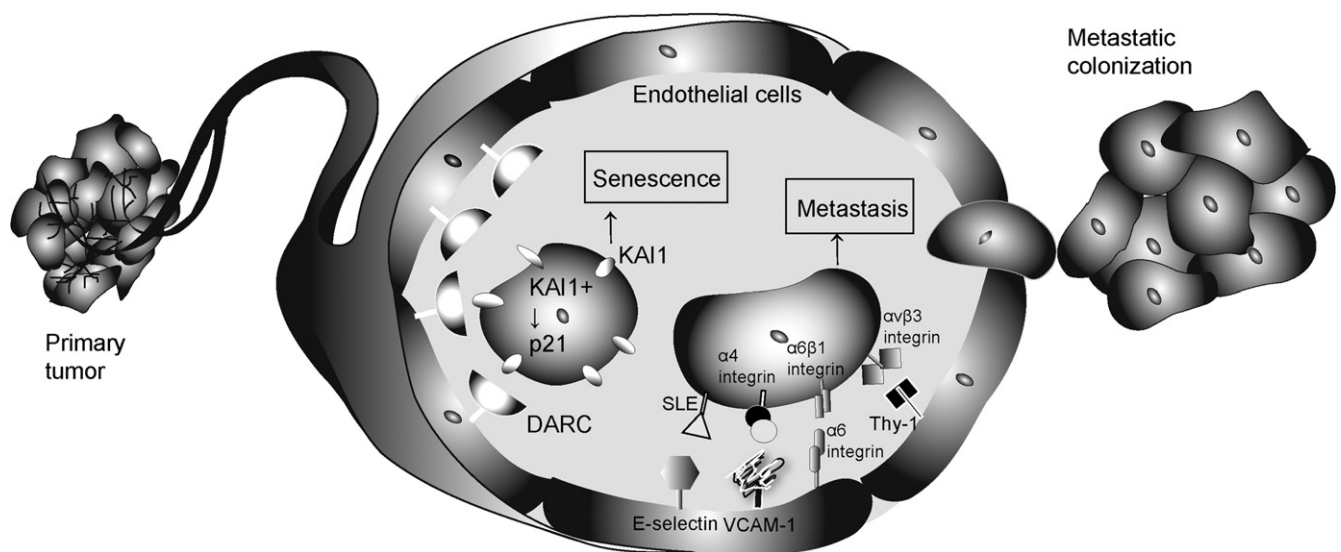

Fig. 1. Interaction of tumor–endothelial cells. Tumor cells in blood vessels attach to endothelial cells through various adhesion proteins. The KAI1 protein on the tumor cell also interacts with DARC on the endothelial cells which induces tumor cell senescence, while a tumor cell without KAI1 successfully extravasates and colonizes in a distant organ.

KAI1 is partly controlled by p53 as reported previously (Mashimo et al., 1998). These results clearly illustrate the importance of interplay between p53, KAI1 and DARC for blocking tumor metastases in the circulatory system.

Signaling of KAI1–DARC interaction

KAI1 is a member of the transmembrane 4 superfamily which is known to form a large complex with multiple proteins which is referred to as tetraspanin web (Rubinstein et al., 1996). KAI1 was indeed found to be associated with many different proteins. Lee et al. recently reported that KAI1 interacts with a tetraspanin, Kitenin, whose over-expression promoted increased tumorigenicity and metastasis *in vivo* (Lee et al., 2004). Kitenin is considered to block the suppressor function of KAI1 which renders the tumor cell more invasive, although the exact molecular mechanism of the Kitenin function is yet to be elucidated. KAI1 was also found to be associated with other tetraspanins, CD9 and CD63 whose functions are not well understood, although these genes are correlated with poor prognosis and increased metastasis (Higashiyama et al., 1995). In addition, Odintsove et al. also reported that KAI1 is able to bind to the EGF receptor and block the EGF-induced cell migration, although the physiological relevance of this observation still needs to be investigated *in vivo* (Odintsova et al., 2000). Like other tetraspanin proteins, KAI1 is also associated with various integrins including $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins (Iiizumi et al., 2007). It should be noted that interaction of KAI1 and $\alpha 3\beta 1$ was found to block the fibronectin/$\alpha 3\beta 1$-induced cell invasion through inhibition of the cytoskeletal system (Zhang et al., 2001). Therefore, KAI1 appears to form a multi-protein complex. However, how this complex affects the interaction of KAI1–DARC and transmits the senescence signal remains to be elucidated. Zhang et al. previously found that KAI1 is also associated with a key signal molecule, protein kinase C (PKC), which controls cell cycle, migration and invasion. It appears that this interaction facilitates recruitment of PKC into proximity with specific integrins such as $\alpha 3\beta 1$ and $\alpha 6\beta 1$ that are known to be strongly associated with KAI1. Therefore, it is plausible that, when KAI1 is activated by DARC on endothelial cells, the KAI1–integrin–PKC complex transduces a signal to induce cell senescence via activation of p21 and TBX2 genes. In this context, it should be noted that PKC is capable of directly controlling p21 expression and activity which is the hallmark of KAI-induced senescence (Deeds et al., 2003). More recently, Sridhar et al. reported that KAI1 is able to suppress integrin-dependent activation of the receptor kinase c-Met *in vitro* (Sridhar and Miranti, 2006). The ligand of c-Met is HGF which is capable of both stimulating and arresting the cell cycle and these effects in either case are mediated through the regulation of p21. Whether this pathway is involved in the KAI/DARC-induced senescence is an interesting question and needs to be clarified.

Therapeutic implication of KAI1–DARC interaction

The ability of DARC to trigger the KAI1 signal which leads to tumor cell senescence suggests an attractive possibility to develop anti-tumor strategy. DARC is known to be a receptor of Plasmodium vivax, a malaria parasite, and more than 70% of West African descendants lack DARC expression and hence are resistant to the infection (Sanger et al., 1955; Tournamille et al., 1995). Epidemiological data indicate that these same populations have significantly higher incidence of various types of cancers as well as higher rate of metastatic disease in comparison to the white population (Luo et al., 2000), suggesting that DARC plays a role in tumor progression. In fact, Shen et al. recently reported that growth of prostate tumor was significantly faster in DARC-deficient mice than in wild type animals (Shen et al., 2006). Wang et al. also found that ectopic expression of DARC in breast cancer cells significantly suppressed the incidence of spontaneous pulmonary metastases in mice (Wang et al., 2006). These observations strongly support the notion that DARC functions as a suppressor for both tumor growth and metastases, and therefore a molecule mimicking the function of DARC may serve as an effective anti-cancer as well as anti-metastatic drug. One approach toward this end would be to define the structure of the interaction domains of KAI1 and DARC, which may provide crucial information to design a small DARC-like peptide capable of engaging in the activation of the KAI1 signal. Another approach is to use chemical libraries to screen compounds that are able to activate the KAI1 signal using senescence markers for the KAI1 activation. These approaches are expected to be effective therapeutic strategy not only for the metastatic disease but also for primary tumors as long as KAI1 is expressed in tumor cells. However, in most patients with metastatic disease, the KAI1 gene is down-regulated although the gene is still intact (Dong et al., 1995). One possible explanation for the down-regulation is the mutations of p53 in these tumor cells. In fact, we have shown that the activation of p53 by etoposide indeed restored the expression of KAI1 *in vitro* (Mashimo et al., 2000), which suggests that it is possible to restore the expression of KAI1 in patients. Therefore, the strategy of restoration of KAI1 expression followed by activation of the KAI1 signal holds a great promise for the treatment of patients even without KAI1 expression. It is expected that further understanding of the nature of KAI1–DARC interaction and its signaling pathway should provide more information regarding other specific targets and strategic options to develop effective anti-metastatic drugs.

Acknowledgments

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